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Spatial variation in soil-borne disease dynamics of a temperate tree, *Prunus serotina*

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Abstract. Soil-borne pathogens are posited to maintain forest diversity. However, their in situ impact and spatial variation are largely unknown. We examined spatial patterns of pathogenic activity in a deciduous forest using a common garden experiment and also in a natural experiment around replicated trees, and we quantified *Pythium* (a soil-borne pathogen) density around individual *Prunus serotina* trees. In both experiments, *P. serotina* seedling survival was 52–57% greater in plots treated with a metalaxyl-based fungicide specific to oomycetes (i.e., *Pythium*) than in untreated plots. Disease dynamics were not density dependent, but pathogenic activity and *Pythium* density were spatially variable. In the common garden and natural experiments, pathogenic activity of soil inoculum varied among trees, while in the natural experiment disease dynamics were also distance dependent and pathogenic activity decreased away from *P. serotina* trees. Disease and *Pythium* density were not always related but displayed considerable spatial variation. We found that *Pythium* density did not vary with distance away from *P. serotina* trees but did vary among trees. Understanding the spatial complexity of soil-borne pathogens is critical to accurately characterizing their effects on populations and ultimately on forest diversity.

Key words: among-tree variability; density-dependent and distance-dependent mortality; Janzen–Connell hypothesis; oomycetes; Prunus serotina; Pythium; *soil-borne pathogens*.

Introduction

Understanding the mechanisms maintaining tree species abundance and diversity in forest communities is a central question in ecology (reviewed in Palmer 1994, Wright 2002, Leigh et al. 2004). The Janzen–Connell hypothesis (Janzen 1970, Connell 1971) predicts that frequency-dependent predation by specialist enemies will prevent a competitively superior species from becoming locally dominant and usurping available resources from subordinate species. Empirical evidence supporting this includes low recruitment success near conspecifics (where seed/seedling densities are generally greatest) and patterns of reciprocal species replacement in temperate and tropical forests (e.g., Woods 1979, Clark and Clark 1984). Moreover, several studies showed that seedling/sapling mortality is greatest near parent trees and/or is density dependent in temperate (e.g., Packer and Clay 2000, Hille Ris Lambers et al. 2002, Masaki and Nakashizuka 2002) and tropical (e.g., Harms et al. 2000, Wills et al. 2006) forests. Although many of these studies speculated on the causes of density- and/or distance-dependent seedling mortality, others specifically implicate soil-borne pathogens (Augspurger 1984, Packer and Clay 2000, Hood et al. 2004, Reinhart et al. 2005, Bell et al. 2006). While it has been argued that these demographic patterns are not strong enough to affect forest diversity (Hubbell 1980), recent research has linked density-dependent sources of mortality to tree diversity in tropical forests (Harms et al. 2000, Wills et al. 2006). Thus, interactions between trees and pathogens may be key factors affecting forest diversity.

In a study elucidating the role of soil-borne pathogens on recruitment success, Packer and Clay (2000) found that seedling mortality of a temperate tree species (black cherry, *Prunus serotina*) was primarily distance dependent, as opposed to density dependent. A parallel greenhouse experiment revealed that *Pythium* pathogens from soil surrounding three *P. serotina* trees caused both distance- and density-dependent mortality (Packer and Clay 2000), supporting the idea that soil pathogens caused distance-dependent mortality in the field. A subsequent, expanded field study found distance-dependent effects on *P. serotina* sapling growth, as well as variation among trees in the strength of distance- and density-dependent effects (Packer and Clay 2002). It should be noted that in many natural systems distance from parent tree and seedling density are correlated.

Although spatial variation in pathogen density and activity has been documented in agricultural environments and model systems (e.g., Burdon and Thrall 1999, Otten et al. 2004), much less is known about the spatial variation of soil-borne pathogens in natural systems

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(Burdon and Chilvers 1982). For example, tree-to-tree variation in plant–soil biota interactions is sometimes accounted for in experiments (e.g., Hood et al. 2004), but has rarely been characterized or acknowledged as ecologically important (but see Van der Putten et al. 1993, Molofsky et al. 2002, Otten and Filipe 2005). Quantifying the impact, density dependence, and density of soil-borne pathogens, as well as their spatial variation, is crucial to understanding the ecological significance of these pathogens.

The objectives of this study were: (1) to explore the natural variation in soil-borne disease dynamics using two in situ field experiments, and (2) to quantify the density of the soil-borne pathogen genus *Pythium* around *P. serotina* trees in a spatially explicit manner. Many *Pythium* spp. are major soil-borne plant pathogens (Hendrix and Campbell 1973), and have been implicated as important seedling pathogens of *P. serotina* (Packer and Clay 2000; see Plate 1). The first field experiment was designed as a common garden experiment to test for the effects of (1) a selective fungicide specific to oomycetes (i.e., *Pythium*), (2) seedling density (low vs. high densities), and (3) soil inoculum collected near multiple trees on seedling survival in a forest understory. The second field experiment, a natural experiment, tested whether pathogenic activity varied with distance from nearest conspecific *P. serotina* tree(s), density of seedlings per plot, per tree, and per site. Finally, we determined the density of *Pythium* isolated from soil samples collected at varying distances away from individual *P. serotina* trees.

Methods

Study system

Our study focused on *Prunus serotina* populations growing in mesophytic deciduous forest in the Indiana University Research and Teaching Preserve system near Bloomington, Indiana, USA. Portions of this research were conducted at the Griffy Lake (39°11′42.65″ N, 86°30′45.59″ W) and Kent Farm (39°8′48.10″ N, 86°23′28.67″ W) sites, which are separated by ~11 km.

Common garden experiment

The goal of this experiment was to test the effects of soil-borne pathogens (fungicide treatment vs. no fungicide), seedling density (low vs. high), and soil inoculum source on the survival of planted *P. serotina* seedlings in a natural forest community. Local seed material was not available in sufficient quantities so the seed used in this experiment originated from Louisiana (Louisiana Forest Seed Company, Lecompte, Louisiana, USA). The Louisiana seed was cold stratified in the lab in sterilized vermiculite ~100 days prior to transplanting. We established the common garden experiment in an area ~3 × 20 m in size in a diverse, mesic deciduous forest on top of a broad ridge at the Griffy Lake site on 10 May 2006. No naturally occurring *P. serotina* seedlings were observed in or immediately around the experimental plots and the nearest mature *P. serotina* trees were >20 m away.

We used a split–split–plot design with seedling density treatments (high vs. low) being nested within fungicide treatments (treated vs. not treated) nested within soil inoculum treatments (soil collected from different *P. serotina* trees or from a control site). The experimental units were replicated twice in two rows consisting of nine paired plots (1 × 0.5 m; $n = 18$ plots per row). Each pair of neighboring plots was inoculated with soil inoculum collected either from around individual trees or from the control common garden location (described below). Thus, the paired plots in each row were randomly assigned nine different inoculum treatments without replacement. One plot per pair was randomly assigned a fungicide treatment (described below) while the other paired plot served as a fungicide control. We then randomly assigned subplots within a plot (0.5 × 0.5 m) either at high or low planting density (described below). Each plot within a row was separated by 0.5 m, and rows were separated by 1 m to limit potential drift of fungicide between plots.

We collected the soil used for inoculating transplanted seedlings from around eight *P. serotina* trees located at the Griffy Lake site and surrounding areas on 9 May 2006. The trees were dispersed over 1 km of forest (or ~27 ha). We collected soil 2 m from the trunk of each tree and to a depth of 10 cm. We collected soil from ~20 points around this circumference and then homogenized all samples from each tree. For the ninth soil sample, an equivalent amount of soil was collected from the common garden area and functioned as a control (i.e., >20 m away from extant *P. serotina* trees).

Recently germinated seedlings of similar age and condition were transplanted into the field and inoculated with field soil treatments in the 25 × 25 cm central portion of a subplot (0.5 × 0.5 m). For each plot, one subplot was planted at high density (20 seedlings per 25 × 25 cm area) and the other at low density (five seedlings per 25 × 25 cm area). We planted seedlings into five locations within a subplot (the center and corners) to control for soil disturbance and amount of inoculum needed per density treatment. In the low density treatment we planted seedlings at one of five random densities per location: 0, 1, 1, 1, or 2. In the high density treatment we planted seedlings at one of five random densities per location: 1, 2, 3, 5, or 9. The density mosaics were representative of natural aggregation patterns occurring in the field at this spatial scale. Five small holes (~5 cm in depth) were made into the soil to accommodate the roots of the seedlings and soil inocula per 25 × 25 cm area. Seedlings were added as described above and each hole was filled with ~50 mL of soil inoculum. To avoid transplant shock, the seedlings were planted during rainy weather, then watered once.

After we planted seedlings and inoculated with field soil, we treated half of the plots with the granular fungicide Subdue GR (active ingredient metalaxyl; Syngenta Crop Protection, Greensboro, North Carolina, USA) at a level of 1.0 g/0.5 m^2 following previously published accounts (Paul et al. 1989, Reinhart et al. 2005). Controls received no fungicide granules. A metalaxyl-based fungicide was selected because it is selective for oomycetes and is used to control *Pythium* and *Phytophora* spp., which cause root rot and damping-off diseases (Schwinn and Staub 1987, Paul et al. 1989; but see Hood et al. 2004). We refer to this chemical as a "fungicide" although the target organisms are not true fungi, and the active ingredient metalaxyl interferes with oomycete development rather than killing the organisms (Schwinn and Staub 1987). The percentage of planted seedlings alive at 30 days was used to quantify seedling survival.

Natural field experiment

This field experiment was designed to test the effect of site, tree, soil-borne pathogens (fungicide vs. no fungicide), initial seedling density (density continuously distributed), and distance to nearest *P. serotina* tree (distance continuously distributed) on survival of naturally emerging *P. serotina* seedlings. We selected five *Prunus serotina* trees each at the Griffy Lake and Kent Farm sites from 20–24 April 2006. The trees were dispersed across 2.2 km (or ~60 ha) at Griffy Lake and 0.25 km (or ~6.25 ha) at Kent Farm.

We initiated the experiment on 20 April 2006, eight days after the first emerging *P. serotina* seedlings were observed. We selected trees with sufficient abundance of *P. serotina* seedlings and established 20 plots scattered around each of the 10 study trees (five trees at each site). Seedling density and distance to parent tree were expected to be correlated. To decouple seedling density and distance, we deliberately selected small plots (50 × 50 cm) so that density of seedlings in the inner plot (inner 25 × 25 cm subplot) and distance to the focal tree varied. However, our sampling was constrained because few high-density plots occurred away from focal trees. As a result, plots were dispersed from 0.7 m to 7.5 m away from the focal trees. Plots were separated from one another by at least 0.5 m to avoid fungicide drift into control plots. To avoid census error, we used small subplots and marked and counted all naturally emerging *P. serotina* seedlings in the inner subplot. Marking seedlings helped control for emergence of additional seedlings. Initial densities ranged from three to 109 seedlings per 25 × 25 cm inner plot. Ten of the 20 plots per tree were randomly selected for treatment with the fungicide Subdue GR, and the other 10 were left untreated as controls. To minimize edge effects around the inner subplot, the entire 50 × 50 cm plot was treated with fungicide at the same level as the common garden experiment (1.0 g/0.5 m^2). We determined seedling survival as the percentage of the initial number of seedlings still alive at 30 days.

Pythium *density around individual trees*

To estimate spatial variation in *Pythium* density in the field, we collected soil from around nine *P. serotina* trees on 21–22 March 2007, before seedlings emerged in the field. Individual trees were dispersed across 2.1 km (or ~57 ha) around the Griffy Lake site with most occurring on separate ridges. Three of the nine *Prunus serotina* trees were previously used in the *Natural field experiment* (see *Methods;* trees 4, 7, and 9). We collected soil cores (0–10 cm in depth) at 2, 4, 8, and 16 m along three transects radiating out from each focal tree. Since microbial populations often vary at small spatial scales (Ettema and Wardle 2002), three soil cores separated by 50 cm were collected at each point and homogenized into a single composite sample ($n = 3$ composite samples per distance per tree). We positioned transects in directions that maximized distance separating transects while maintaining the distance intervals away from other *P. serotina* trees. This ensured that soil samples collected at a fixed distance (e.g., 16 m) away from the focal tree remained at least that distance away from the nearest *P. serotina* tree.

We determined *Pythium* density using the plate dilution-frequency (most probable number) technique (Lumsden et al. 1975). First we air dried the field soil for one week. Weighed samples of dried soil (~2 g) were added to 50 mL of dilute agar (2.5 g of agar per liter water) and mixed on a shaker for 10 minutes. After mixing, we performed a serial dilution using test tubes filled with dilute water agar. Plates were established according to Lumsden et al. (1975) on selective growth media (P_5ARP; Martin 1992). Although the media is selective for oomycetes and against most soil microbes, one common fungal saprobe (*Mortierella*) did grow on the selective P_5ARP media. Previous work culturing isolates of *Pythium* from a range of forest soils also resulted in cultivation of *Mortierella*, which was confirmed by DNA sequencing (*unpublished manuscript*). Independent trials growing the two genera side by side revealed consistent differences in morphology and colony growth rates that were used to distinguish and discard *Mortierella* cultures. After incubation in the dark at 25°C for 24 hours, the number of droplets with *Pythium* per dilution step was counted. Quantifying colonies after only 24 hours precluded other slower growing oomycetes such as *Phytophthora*. The total number of droplets in the series with *Pythium* was converted into the number of propagules per gram of air-dried soil by reference to a standard table (Harris and Sommers 1968).

Statistical analysis

We fit the data from the *Common garden experiment* with a generalized linear mixed model using Proc

TABLE 1. Effect of fungicide, seedling density (five or 20 per plot), and soil inoculum source (samples from eight *Prunus serotina* trees) on final counts of living *P. serotina* seedlings per planted seedling density for the "common garden" field experiment.

Fixed effects	df	*F*	*P*
Fungicide	1, 7	8.74	**0.021**
Density	1, 46	4.06	**0.050**
Fungicide × density	1, 46	0.03	0.860
Random effects	df	χ^2	*P*
Inoculum	1	13.50	**0.0001**
Fungicide × inoculum	1	3.53	**0.030**

Notes: Inference tests are shown for fixed and random effects. Data were fit with a general linear mixed model, and random effects were tested with the likelihood ratio statistic. Fungicide was used against the soil-borne pathogen *Pythium*. Significant results are shown in boldface type. See *Methods: Common garden experiment* for further details.

GLIMMIX in SAS version 9.2 (SAS Institute 2007). A binomial distribution and a logit function were used since data represent the number of dead seedlings out of either five or 20. Laplace was selected as the optimization method (Schabenberger 2007). The analysis did not include data relating to the control inoculum collected away from *Prunus* because of insufficient sample size ($n = 1$), and data are shown in Appendix A only for visual comparison. The analysis was performed using seedling density, fungicide, inoculum source (i.e., trees), density × fungicide, and inoculum source × fungicide as variables. Density and fungicide were treated as fixed effects. Random effects included inoculum source and inoculum source × fungicide. Inference tests of the random effects (inoculum source and inoculum source × fungicide) were performed using the likelihood ratio statistics provided by the covtest statement in Proc GLIMMIX.

We fit the data from the *Natural field experiment* (see *Methods*) with a generalized linear mixed model using Proc GLIMMIX in SAS similar to that described previously. Fixed effects included site, fungicide, density, distance, site × fungicide, fungicide × density, and fungicide × distance. The random effects included tree (site) and fungicide × tree (site). Inference tests of the random effect (i.e., fungicide × tree) were performed using the likelihood ratio statistics provided by the covtest statement in Proc GLIMMIX. The density data had a constrained distribution consistent with the "envelope effect" (i.e., variation in survival is large at low densities but smaller at high densities where survival was consistently low; sensu, Goldberg and Scheiner 2001). The data had a constrained distribution because we could not fully separate seedling density from plot distance away from the parent tree since they were correlated. We identified and removed plots if they had densities that exceeded three standard deviations (>61 seedlings per plot). They were removed to avoid including plots with extreme densities that had strong effects on the best fit regression lines shown in figures. Based on this criterion, we removed two fungicide-treated plots (densities of 64 and 82 seedlings per plot) and two untreated plots (65 and 109 seedlings per plot) from the data for the Kent Farm site.

We fit the *Pythium* density data with a mixed model using Proc Mixed in SAS. Distance was treated as a fixed effect and tree, tree × distance, transect (tree), distance × transect (tree) as random effects. Degrees of freedom were estimated using the Kenward-Roger's method (Littell et al. 2002). *Pythium* density was log-transformed. To determine if *Pythium* density varied among trees, the density data from immediately around *Prunus serotina* trees (2 m and 4 m) were fit with an identical mixed model. Inference tests of the random variables "tree" and "tree × distance" were performed by calculating the likelihood ratio statistics (see Littell et al. 1996).

RESULTS

Common garden experiment

Averaged over all inoculum sources, fungicide applications increased seedling survival 57% relative to untreated controls, ($P = 0.021$; Table 1, Fig. 1). Increasing planting density had a negative effect on seedling survival ($P = 0.050$; Table 1, Fig. 1). However, the interaction between planting density and fungicide treatments, which would indicate if disease dynamics were density dependent, was not statistically significant ($P = 0.86$; Table 1). There was a significant effect of soil inoculum source (i.e., tree where soil was collected) on seedling survival ($P = 0.0001$; Table 1), and an interaction between fungicide and inoculum source on seedling survival ($P < 0.0001$; Table 1; Appendix A).

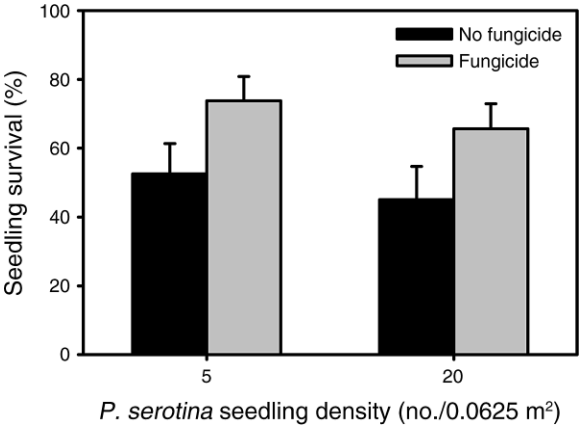

FIG. 1. Effect of selective fungicide treatments against the soil-borne pathogen *Pythium* and seedling density on the survival of *Prunus serotina* seedlings ($n = 16$ per treatment). Values are means + SE. There was a statistically significant effect of fungicide ($P = 0.021$), a negative effect of planting density ($P = 0.050$), and no interactive effect of density and fungicide ($P = 0.86$). Appendix A illustrates how the effects of fungicide varied among trees and includes the "away from *Prunus*" data.

TABLE 2. Effect of site, fungicide, density of emerging *Prunus serotina* seedlings, plot distance away from focal *P. serotina* trees, and two-way interactions (e.g., fungicide × tree) on counts of surviving *P. serotina* seedlings/initial seedling density for the "natural" field experiment.

Fixed effects	df	*F*	*P*
Site	1, 8	0.57	0.47
Fungicide	1, 8	25.86	**0.0009**
Density	1, 171	65.71	**<0.0001**
Distance	1, 171	29.65	**<0.0001**
Site × fungicide	1, 8	6.33	**0.036**
Fungicide × density	1, 171	0.13	0.72
Fungicide × distance	1, 171	19.76	**<0.0001**
Random effects	df	χ^2	*P*
Fungicide × tree	1	3.22	**0.036**

Notes: Data were fit with a general linear mixed model, and random effects were tested with the likelihood ratio statistic. Significant results are shown in boldface type. See *Methods*: *Natural field experiment* for further details.

This indicates that certain trees harbored more virulent inoculum, and fungicide treatment greatly enhanced seedling survival, while inoculum from other trees was relatively avirulent and fungicide treatment had little effect.

Natural field experiment

Damping-off disease symptoms, typically associated with soil-borne pathogens, were prevalent during the course of the field experiments at both experimental sites. Seedling survival increased on average by 52% with fungicide applications ($P = 0.0009$; Table 2). The beneficial effect of fungicide varied among trees ($P = 0.036$) and between sites ($P = 0.036$; Table 2, Fig. 2). There was also a significant positive effect of distance on seedling survival ($P < 0.0001$), and the benefit of fungicide on seedling survival decreased with increasing distance from focal *Prunus serotina* trees ($P < 0.0001$; Table 2, Fig. 3A). Seedling survival was negatively affected by the density of *P. serotina* seedlings ($P < 0.0001$; Table 2, Fig. 3B). However, the interaction between density and fungicide treatments, indicating whether disease dynamics were density dependent, was not statistically significant ($P = 0.72$; Table 2).

Pythium *density around trees*

Pythium density did not vary with distance away from *Prunus serotina* trees (ANOVA, $F_{3,96} = 0.42$, $P = 0.74$), and there was no effect of tree × distance (likelihood ratio statistic, $\chi^2 = 0$, df = 1, $P = 1.0$; Appendix B). In a second analysis to look at variation among trees using data from the two distances nearest the focal *P. serotina* trees (2 m and 4 m), *Pythium* density was found to vary among trees (likelihood ratio statistic, $\chi^2 = 13.8$, df = 1, $P = 0.0002$) with as few as 5.1 ± 3.1 propagules per gram of soil (mean ± SE) to as many as 517.5 ± 150.9 (Fig. 4).

DISCUSSION

Spatial variation of disease

Additions of selective fungicide increased seedling survival by 52–57% for the two field experiments, indicating that soil-borne oomycete pathogens cause significant mortality of *Prunus serotina* seedlings in nature. These estimates of disease caused by oomycetes are likely to be conservative because of the experimental design (e.g., timing and amount of fungicide application) and limits of biocide efficacy. Although the average benefit of adding fungicide was relatively constant across experiments, pathogenic activity varied considerably at multiple spatial scales. For example, pathogenic activity, as measured by seedling survival in plots treated with fungicide vs. untreated plots, was concentrated around *P. serotina* trees and diminished while moving out to 8 m away (Fig. 3A). This pattern suggests that seedling mortality caused by soil-borne oomycetes is distance dependent. However, the density of *Pythium*, as estimated by dilution plating, did not vary with the

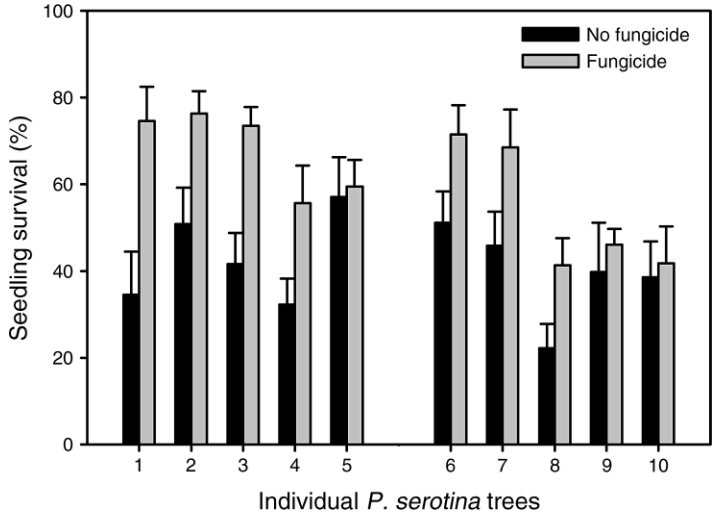

FIG. 2. Effect of selective fungicide treatment on the survival of *Prunus serotina* seedlings among trees ($n = 10$) where plots were established. Trees where plots were established had relatively high numbers of seedlings relative to other conspecific trees at each site. Trees 1–5 were from the Griffy Lake site, and trees 6–10 were from the Kent Farm site. Means (+SE) are based on the means across plots ($n = 8$–10 per treatment per tree) with data shown in Fig. 3. Tree numbers are arbitrary and do not correspond with those in other figures. There was an interaction between fungicide and tree on survival ($P = 0.036$) and an interactive effect of fungicide and site ($P = 0.036$).

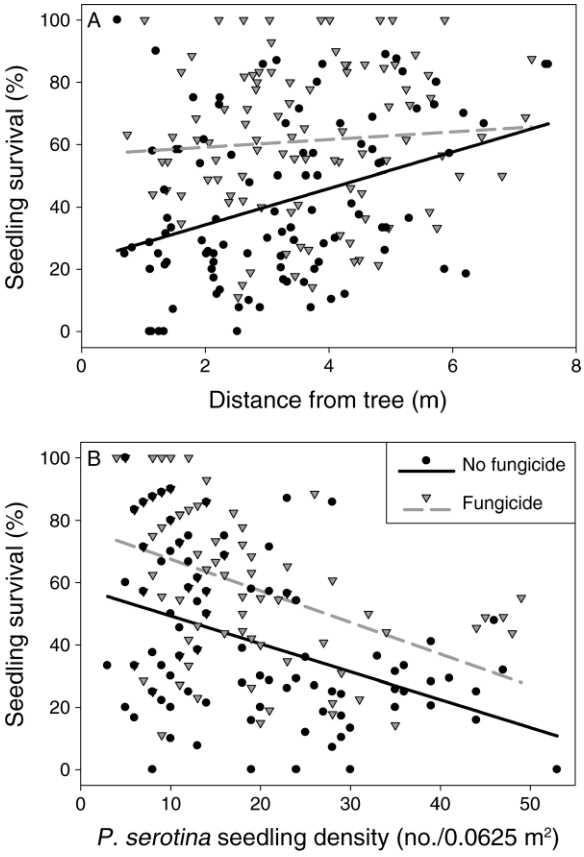

FIG. 3. Effect of selective fungicide treatment on the survival of *Prunus serotina* seedlings in relation to (A) plot distance away from *P. serotina* tree and (B) natural emergence density of *P. serotina* seedlings per plot. Twenty plots (10 treated and 10 untreated) were established around five *P. serotina* trees at each of two sites ($n = 97$–98 plots per fungicide treatment). Best-fit linear regression lines are shown. Radial patterns at low densities in panel (B) are driven by the limited potential responses of plots with low densities (<20 seedlings per plot). (A) There was an interactive effect of fungicide and distance on seedling survival ($P < 0.0001$). (B) There was no interactive effect of fungicide and seedling density on survival ($P = 0.72$). Fig. 2 illustrates how the effects of fungicide varied among trees and sites.

distance (2, 4, 8, and 16 m) that soil samples were collected from *P. serotina* trees (Appendix B).

We found that pathogenic activity and *Pythium* density varied considerably among individual trees and sites (Figs. 2 and 4, and Appendix A). Tree-to-tree variation was also documented in an earlier study of seedling and sapling growth around six *P. serotina* trees (Packer and Clay 2002). Tree-to-tree variation may be caused by fluctuations in the relative abundance or activity of oomycetes in general, or by the spatial variability in the density of specific pathogenic *Pythium* or other oomycete species. Tree-to-tree variation may also reflect environmental differences (e.g., soil type), dispersal limitation by the pathogen, and/or biological differences among individual trees (e.g., age, secondary chemistry, species composition of neighbors). Although our work helps illuminate the "black box" of soil microbial communities, more work is needed to determine what levels of spatial variation and specific identities are important in understanding the pathogen landscape (reviewed in Ettema and Wardle 2002).

Disease and pathogen density

The apparent contradiction between the distance-dependent disease dynamics caused by oomycete pathogens and lack of spatial patterning in the density of *Pythium* with distance from trees suggests that we may not be accurately measuring the disease-causing pathogen(s). Individual *Pythium* species are known for having intermediate levels of specificity (i.e., cause mortality in more than one, but not all species), and can vary from strong to weak pathogens for a given host species (Augspurger and Wilkinson 2007; K. O. Reinhart, T. Tytgat, W. H. Van der Putten, and K. Clay, *unpublished manuscript*). Our quantification of total *Pythium* density combined densities of all *Pythium* species including both virulent and avirulent species. Avirulent *Pythium* may function as saprobes. If saprophytic *Pythium* are common or ubiquitous then they could obscure distance-dependent patterns of virulent *Pythium* species. Future studies should attempt to determine which species are important pathogens of *P. serotina* (e.g., *Py. attrantheridium*/*intermedium* and *Py. sylvaticum*; K. O. Reinhart, T. Tytgat, W. H. Van der Putten, and K. Clay, *unpublished manuscript*) and to quantify their individual densities around *P. serotina* trees. This will require methodologies like quantitative PCR combined with pathogenicity experiments with individual isolates. The potential pathogenic effects of related oomycetes (e.g., *Phytophthora* spp.) should also be enumerated since they would also be suppressed by fungicide treatment.

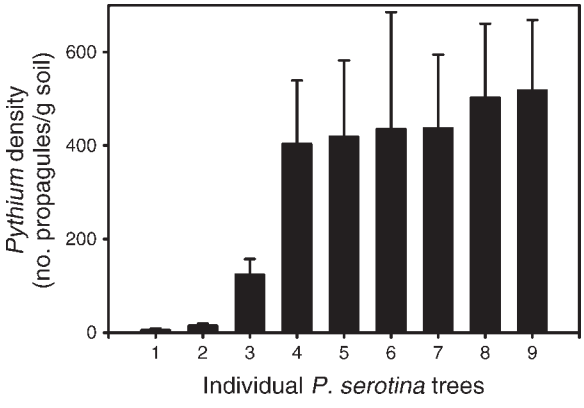

FIG. 4. Densities of *Pythium*, a soil-borne pathogen, varied among *Prunus serotina* trees ($P = 0.0002$). Densities are based only on data for soil sampled near the focal tree (at 2 m and 4 m; $n = 6$ soil samples per tree). Tree numbers correspond to those used in Appendix B (not those in Fig. 2), showing data for all trees and four sampling distances away from the trees. Data are means + SE.

PLATE 1. Each spring, vast numbers of *Prunus serotina* seedlings emerge in Indiana forests, and many are decimated by disease caused by soil-borne pathogens. Left of center in the photograph is a recently emerged *P. serotina* seedling that has relented to damping-off disease, likely caused by the oomycete *Pythium*. Right of center is a *P. serotina* seedling that appears unaffected. Photo credit: K. O. Reinhart.

Density-dependent disease

Using a field experiment, Bell et al. (2006) reported that soil-borne pathogens caused density-dependent mortality of a tropical tree species, but their study did not test for distance-dependent sources of pathogenic activity. Our experiments looked for both distance- and density-dependent sources of mortality caused by soil-borne pathogens, which can be challenging to decouple because they are naturally correlated (Freckleton and Lewis 2006). Although density had a direct negative effect on seedling survival, we did not observe an interaction between fungicide treatment and seedling density for the two field experiments. Distance could not be unambiguously decoupled from density in the natural field experiment. Also supporting a primary role for distance dependence, pathogenic activity was not density dependent in the common garden experiment where seedling density was experimentally manipulated. Density-dependent disease dynamics are widely cited in the literature (reviewed in Burdon and Chilvers 1982), and Packer and Clay (2000) found that seedling density was a significant determinant of survival of *P. serotina* seedlings and interacted with distance during a laboratory experiment. However, the two in situ field experiments here did not detect density-dependent disease dynamics but did reveal distance dependence, corresponding to the demographic patterns reported by Packer and Clay (2000).

Our results from two field experiments are interesting because distance from parent trees and seed densities are typically negatively correlated (e.g., Smith 1975), yet only one of the two factors (distance) was found to interact with fungicide. One possibility for this result is that researchers quantify density in the field differently than soil-borne pathogens perceive it. For example, our a priori prediction was that plots with high seedling density would have greater pathogenic activity than those with low density. However, plots with low numbers of naturally emerging seedlings may be associated with greater pathogenic activity because *Pythium* pathogens can cause both pre- and post-emergence mortality. Low density plots, therefore, might be the ones with high pre-emergence disease mortality and high densities of pathogen inoculum. However, the common garden field experiment, which demonstrated no interaction between fungicide and planting density, experimentally manipulated the planting density of seedlings and was therefore not confounded by pre-emergence mortality. Further, the common garden field experiment was established in an area with no recent legacy of *P. serotina* trees.

Other factors affecting pathogenic activity

Prior years of prolific seed production and seedling emergence may cause *Pythium* propagules to build up in the soil (Packer and Clay 2004). Host–pathogen legacies, which are more complicated to quantify than seed/seedling density at a single point in time, might help account for the spatial variation in pathogenic activity and *Pythium* density reported here. *Pythium* spp. also infect the roots of saplings and trees (Packer and Clay 2002). Therefore, established trees themselves may help maintain *Pythium* populations, limiting recruitment by conspecific seedlings. Synchronized emergence of seedlings (K. O. Reinhart, *personal observation*) and fine root proliferation by established trees and saplings may serve as important resource fluxes that affect *Pythium* populations. Moreover, many *Pythium* spp. can function as saprobes for portions of their life cycle, and their

density may fluctuate with pulses of detritus (Martin and Loper 1999). This is complicated further because *Pythium* spp. can cause disease in multiple species (e.g., Augspurger and Wilkinson 2007). Thus, the density of seedlings of a single species is likely only one of many drivers affecting the density of *Pythium* in the soil.

Host specificity

A major assumption of the Janzen–Connell hypothesis is that predators or pathogens are host specific and do not affect all species equally. Our study did not attempt to determine the specificity of the pathogens, although previous work suggests that there is one or more *Pythium* spp. relatively specific to *Prunus serotina* (Packer and Clay 2000) and that *P. serotina* is more susceptible to damping-off disease than some other temperate tree species (K. O. Reinhart, D. Johnson, and K. Clay, *unpublished manuscript*). The assumption that all enemies are highly specific is not supported by two recent studies suggesting that *Pythium* (Augspurger and Wilkinson 2007) and a range of foliar fungal pathogens have intermediate levels of host specificity (Gilbert and Webb 2007). Predators and pathogens with intermediate levels of specificity may be prevalent in natural systems. In the case of the oomycete pathogens described here, if their hosts become more abundant, then the pathogen should become more prevalent and more homogenously distributed (unless affected by spatial variation in abiotic factors or indirect biological interactions). Increases in the prevalence of the pathogen will subsequently limit the ability of its host(s) to maintain dominance. Thus, variation in the frequencies and spatial distributions of hosts and enemies may help maintain species coexistence and plant diversity across the landscape without requiring enemies that are highly host specific.

Spatial distribution of Prunus serotina

The Janzen–Connell hypothesis also suggests that host-specific parasites and pathogens may generate hyperdispersion of adult trees, and help to maintain forest diversity by constraining competitive dominance by particular species. Our results support the general framework of the Janzen–Connell hypothesis by demonstrating that soil-borne *Pythium* pathogens can reduce seedling survival in a distance-dependent manner. However, our results also demonstrate considerable heterogeneity among trees in terms of both the virulence of soil inoculum and the density of *Pythium* isolated from soil samples (Figs. 2 and 4, and Appendix A). This pattern could result in strong limitation of seedling establishment in some parts of the forest but little constraint on seedling establishment in other areas. It would be useful to more closely examine the spatial distribution of *Prunus* in local forests, and to determine how the patterns demonstrated here vary with spatial structure of mature trees. Our results clearly demonstrate that *Pythium* pathogens can provide a mechanism generating Janzen–Connell-type patterns, but that other factors create a more complex biological landscape.

Implications for future research

Our research has important implications for empirical plant–soil biota interaction studies and the general assumptions of plant–soil biota feedback models. The spatial variation around and among individual trees has important implications for empirical studies designed to characterize plant–soil biota interactions. Recent plant–soil biota studies are generally accompanied by two types of experiments and their hybrids: inoculum/filtrate experiments and soil feedback experiments (Bever 2002; reviewed in Reinhart and Callaway 2006). Both types generally rely on sampling designs that collect multiple soil samples either systematically (e.g., at fixed distances away from a focal species) or randomly (e.g., multiple rhizosphere samples collected in a given habitat). Our results indicate that soil sampling should be designed to account for natural variation, and independent samples should be kept distinct unless pooling samples is well-justified. In many experiments, soil samples that are initially distinct are homogenized into a single composite sample. Subsamples from the composite lack independence from one another (pseudoreplication), which complicates relating the results back to a specific segment of the system (i.e., soil is no longer from one plot or near one tree; Scheiner 2001). Another concern is that it is unclear whether the average effect size and direction would be similar using multiple independent samples (i.e., multiple individual soil communities) vs. a composite sample.

Our research also has important implications for the general assumptions of plant–soil biota feedback models. Several studies have evaluated the strengths and directions of plant–soil biota feedbacks (e.g., Bever et al. 1997, Bever 2002, 2003). The feedback approach posits that plants affect organisms in their rhizospheres, and that rhizosphere biota in turn affect the plants. The direction of the feedback depends on the relative negative effects of accumulating soil antagonists vs. the relative positive effects of accumulating soil-borne mutualists. For example, host-specific soil-borne pathogens (*Pythium* spp.) were found to accumulate in association with two grasses (*Danthonia* and *Panicum*) more than a third grass (*Anthoxanthum*; Mills and Bever 1998). Although the feedbacks are believed to trigger the proliferation of some microbes over others, the spatial considerations of the feedback model are often relatively simple and assume that the plants and soil microbes at the start of the feedback are well-mixed and well-dispersed, which may not be the case as we show here (see Figs. 2 and 4, and Appendix A). The soil biota associated with the trees in our study, because of their age as mature trees, are likely to be associated with soil biota that approaches the community at the theoretical feedback endpoint (i.e., soil feedbacks are believed to be dynamic with the microbes responding to the presence of

a host species and should stabilize over time until the host dies). If well-mixed, then the species pools associated with different trees should be relatively similar and the plant–soil biota interactions should converge as the feedbacks proceed through time. However, our study revealed considerable variation around and among trees, suggesting that species pools used to establish the soil feedback may differ among trees and that they are not as well-mixed as previously thought.

Conclusions

Exploring the spatial nature of disease in relation to the spatial variation of hosts has largely been ignored in past studies. Although a number of studies have used demographic patterns and descriptions of disease to conclude that soil-borne pathogens are driving recruitment patterns (e.g., Augspurger 1984, Forget 1997, Packer and Clay 2000), fewer have tested the Janzen–Connell hypothesis by quantifying the effect of soil-borne pathogens using controlled field experiments (but see Dalling et al. 1998, Bell et al. 2006). For example, Bell et al. (2006) demonstrated significant density-dependent disease dynamics for a tropical tree species. In contrast, we found no evidence of density-dependent disease dynamics for a temperate tree. Instead *Pythium* density and disease exhibited considerable spatial variation across sites and pathogenic effects were primarily attributable to distance-dependent effects. Because *Pythium* species are not highly host specific, greenhouse experiments that manipulate host density in isolation from other potential hosts are likely to report density-dependent effects, while patterns in nature are likely to be more complex. The plant pathology literature has predominately focused on row crops where planting densities are essentially fixed (e.g., Burdon and Chilvers 1982). Incorporating spatial variation into future plant–soil biota experimental designs and models is critical to validate the importance of these interactions in maintaining species diversity in natural communities.

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APPENDIX A

Effect of selective fungicide treatment and source of soil inocula from one of eight *Prunus serotina* trees or from soil collected ≥20 m away from the nearest *Prunus serotina* tree (*Ecological Archives* E090-214-A1).

APPENDIX B

Pythium density of soil samples collected around nine *Prunus serotina* trees (*Ecological Archives* E090-214-A2).